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Glu 69 is essential for the high sensitivity of muscle fructose-1,6-bisphosphatase inhibition by calcium ions

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Abstract Muscle fructose-1,6-bisphosphatase (FBPase) is highly sensitive toward inhibition by AMP and calcium ions. In allosteric inhibition by AMP, a loop 52–72 plays a decisive role. This loop is a highly conservative region in muscle and liver FBPases. It is feasible that the same region is involved in the inhibition by calcium ions. To test this hypothesis, chemical modification, limited proteolysis and site directed mutagenesis Glu^{69}/Gln were employed. The chemical modification of Lys$^{71–72}$ and the proteolytic cleavage of the loop resulted in the significant decrease of the muscle FBPase sensitivity toward inhibition by calcium ions. The mutation of Glu^{69} → Gln resulted in a 500-fold increase of muscle isozyme $I_{0.5}$ vs. calcium ions. These results demonstrate the key role that the 52–72 amino acid loop plays in determining the sensitivity of FBPase to inhibition by AMP and calcium ions.

Keywords: Calcium; Glyconeogenesis; Muscle fructose-1,6-bisphosphatase; Site-directed mutagenesis

1. Introduction

Fructose-1,6-bisphosphatase (FBPase) catalyses the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate and orthophosphate in the presence of divalent cations such as magnesium, manganese or zinc [1–3]. Both muscle and liver FBPase isozymes have been discovered in mammalian tissues. The former participates in glycogen synthesis from lactate (glyconeogenesis), the latter is a regulatory enzyme involved in the gluconeogenesis. The kinetic properties of both isozymes are virtually identical: both are inhibited competitively by fructose-2,6-bisphosphate and allosterically by AMP [4–6]. The fundamental difference between the liver and the muscle isozyme is their sensitivity toward AMP inhibition. $I_{0.5}$ of the muscle isozyme is in the range of 0.05–1 μM, whilst the corresponding value of the liver isozyme is in the range of 5–15 μM [3,6].

Mammalian FBPases are homotetrameric enzymes [7]. The tertiary structure of each monomer is composed of two domains: the Fru-1,6-P_2 domain containing the active site and the AMP binding domain with the AMP binding site. The metal binding site is located between the Fru-1,6-P_2 and AMP binding domains [8,9]. The mammalian liver FBPase exists at least in two quaternary conformations called R and T depending on the relative concentrations of the enzyme effectors [8,10]. The mammalian liver FBPase consists of the upper dimer (C1 and C2) and the lower dimer (C3 and C4). It has been shown that the communication between the subunits within the dimers is possible by interaction of 7–15 N–terminal residues and the loop 187–196 of C1 with the loop 52–72 of C2. A proposed mechanism for the allosteric regulation of catalysis involves three conformational states of the loop 52–72 called: engaged, disengaged and disordered. FBPase is active when the loop 52–72 oscillates between the engaged and disordered state, and inactive or less active when the loop 52–72 is stabilized in its disengaged conformation [11]. Although the crystallographic study of muscle FBPase has been reported, no details concerning the conformation of this isozyme have been released [12]. Nevertheless, it seems reasonable to assume that the mammalian muscle isozyme also exists in conformations R and T and the similar mechanism for allosteric regulation includes three conformational states of the loop 52–72: engaged, disengaged, and disordered.

Recently, we have reported [13] that the muscle FBPase, unlike the liver isozyme, is strongly inhibited by calcium ions. The determined $I_{0.5}$ was ca. 0.6 μM. We have also found that calcium causes the dissociation of FBPase aldolase complex, which is necessary for glyconeogenesis to proceed and the dissociation of FBPase from the Z-line [14]. On the basis of our results we have postulated that calcium ions regulate glyconeogenesis.

Searching for the origin of the high sensitivity of muscle FBPase toward calcium ions the chemical modification with fluorescein and tetramethyl-rhodamine isothiocyanate (FITC and TRITC), proteolytic modification with subtilisin, and site-directed mutagenesis was employed.

The results of our investigation lead us to conclusion that the loop 52–72 is critical for the inhibition of the muscle FBPase by calcium and that the Glu^{69} is essential for the high affinity of the enzyme toward calcium ions.

2. Materials and methods

2.1. Enzyme purification and activity determination

FBPase was isolated from the rabbit muscle according to the method previously described [6]. The FBPase concentration was determined spectrophotometrically at $\lambda = 280$ nm, assuming that $A^{1\%}_{1\ cm} = 6.3$. The purified enzyme was checked for its homogeneity on SDS–PAGE, as previously described [15].

2.2. Kinetic studies

Kinetic experiments were performed at pH 7.5 and 37 °C as previously described [16]. One milliliter of the assay mixture contained:

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Table 1
The region 50–80 of mammalian muscle and liver FBPases

Source	Muscle isozyme	Liver isozyme
Man	kaglahlygi agsvnvtgde vkkldvlsnsl	kagiahlygi agstnvtgdq vkkldvlsndl
Rat	kaglanlygi agsvnvtgde vkkldvlsnsl	qagiaqlyg iagstnvtgdq vkkldilsndl
Mouse	kaglanlygi sgsvnvtgde vkkldvlsnsl	kagiahlygi agstnvtgdq vkkldvlsndl
Rabbit	kaglahlygi agtvnvtgde vkkldvlsnal	kagiahlygi agstnvtgdq vkkldvlsndl

The loop 52–72 is underlined.

50 mM BTP, 2 mM $MgCl_2$, 150 mM KCl, 1 mM EDTA, 0.2 mM NADP, 20 units glucose-6-phosphate dehydrogenase, 10 units glucose-6-phosphate isomerase and various concentrations of Fru-1,6-P_2. The substrate was used to start the reaction. One unit of enzyme activity is defined as the amount of the enzyme that catalyses the formation of 1 μmol of product per minute. Spectrophotometric measurements were performed with a HP 8452A diode array spectrophotometer. The determination of $I_{0.5}$ and Hill coefficient for: AMP and Ca^{+2} as well as $A_{0.5}$ and Hill coefficient for Mg^{2+} were performed using the GraFit 3 programme [17]. All other kinetic parameters such as: K_m, K_i for Fru-2,6-P_2 and K_{is} for Fru-1,6-P_2 (with its standard error) were calculated with Excel and GraFit assuming the uncompetitive inhibition of the enzyme by the substrate [6]. The AMP concentration was determined spectrophotometrically using 15400 M^{-1}/cm^{-1} as the molar absorption coefficient at 260 nm. To measure Fru-2,6-P_2 concentration, this compound was hydrolyzed with HCl at pH 2.0 for 10 min and fructose 6-phosphate was determined as previously described [16].

2.3. Fluorescent labeling

Fluorescently labeled FBPases were obtained by the modification of the proteins with FITC and TRITC [18]. The lack of the proteolysis of fluorescently labeled proteins was checked by 10% SDS–PAGE [15]. The number of the fluorochrome molecules conjugated to the enzymes was determined spectrophotometrically. During the labeling (5 h) the sample was incubated at 4 °C and dialyzed extensively against PBS pH 7.4.

2.4. Subtilisin cleavage

The sample of FBPase was incubated for 3 h at 37 °C with the subtilisin in the molar ratio 100:1 in the BTP/HCl buffer pH 7.5, containing $MgCl_2$, EDTA and KCl, as described for kinetic studies.

2.5. Site-directed mutagenesis

The mutagenesis of the muscle and liver FBPase was performed using the Altered Sites®II *in vitro* Mutagenesis System (Promega) according to the protocol provided. First, an *Eco*RI–*Pst*I fragments encoding the N-terminal 190 bp sequence of muscle FBPase gene and the complete 1070 bp sequence of liver FBPase gene were obtained by the digestion of pHMF2 and pJT2 plasmids [19]. Then these two genes were cloned into the pALTER vector supplied by the manufacturer. Next, to generate the $Glu^{69} \rightarrow Gln$ mutation in the muscle FBPase and the $Gln^{69} \rightarrow Glu$ mutation in the liver FBPase, the resulting pED4 (muscle FBPase) and pED2 (liver FBPase) plasmids were mutagenized with primers MEQ69: 5′-phospho-CGTGACGGGAGATCAGGTGA-AGAAACTG-3′ and LQE69: 5′-phospho-CCAACGTGACAGGT-GATGAGGTTAAGAAGCTGGACC-3′ (mutations underlined), respectively. Finally, the mutations were confirmed by sequencing and the mutated *Eco*RI–*Pst*I fragments of muscle and liver FBPase were recloned into the pHMF2 and pJT2 expression plasmids based on pKK223-3 (Pharmacia Biotech) to form pEQ69 (muscle $Glu^{69} \rightarrow Gln$ FBPase) and pQE69 (liver $Gln^{69} \rightarrow Glu$ FBPase) plasmids.

2.6. Expression and purification of wild-type and mutant FBPases

The purification protocol was similar to the former, describing the isolation of FBPase from the rabbit muscle. The 2.5 L bacterial culture was collected by the centrifugation. The remaining pellet was suspended in BugBuster (Prospecta) solution 1 g: 5 ml for homogenization, left in the room temperature for 20 min and centrifuged. The succeeding steps were taken as described for the purification of the rabbit muscle FBPase. The supernatant was collected and fractionated with ammonium sulfate, and the affinity chromatography on phospho-cellulose was employed as described.

3. Results and discussion

It has been previously reported that calcium is rather a weak inhibitor of the muscle FBPase [20]. We have found recently that calcium is the strong inhibitor of the muscle FBPase and that proteolysis decreases the sensitivity of the enzyme toward calcium [13]. Perhaps the reported low affinity of the muscle FBPase to calcium ions was due to a partial proteolysis of the enzyme.

The mechanism of the muscle FBPase inhibition by calcium is unknown. The interaction of calcium with FBPase may induce conformational changes of the enzyme stabilizing the loop 52–72 in the disengaged position thus resulting in an inactive or less active isozyme form. To test this hypothesis we employed the chemical modification of FBPase with FITC and TRITC in order to introduce fluorescein or tetramethyl-rhodamine to ε-amino group of the exposed lysines. The determined stoichiometry of the modification with FITC was 1.8 fluorescein molecule per tetramer of FBPase and with the use of TRITC it was 2.8 tetramethyl-rhodamine molecule per FBPase tetramer. To determine the site of fluorescent labels binding the mass spectrometry was employed. The labeled protein was electrophorized on SDS 10% polyacrylamide gels and protein bands were investigated with ESI MS (electrospray ionization mass spectrometry). The identified tryptic peptides marked with fluorescent labels belonged to the regions: $K^{71–72}$, K^{141}, $K^{204–205}$ and C-terminal peptide.

In Table 1 the region 49–78 (containing $Lys^{71–72}$) of muscle and liver FBPases is presented. The relatively large group (400–500 Da) of fluorescein or tatramethyl-rhodamine bound

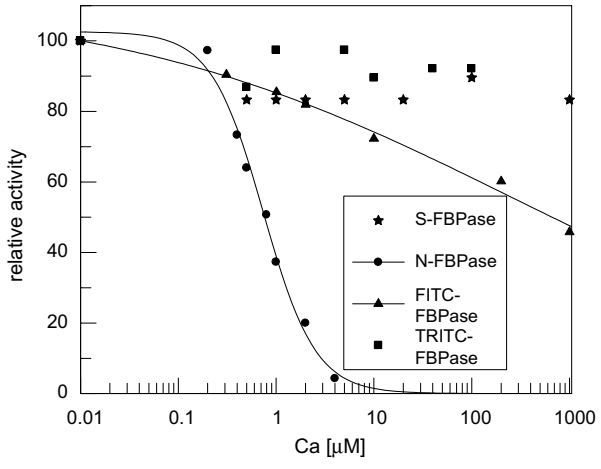

Fig. 1. The effect of calcium ions on the activity of the native rabbit muscle FBPase (N-FBPase) and the enzyme modified with fluorescein isothiocyanate (FITC-FBPase), tetramethyl-rhodamine isothiocyanate (TRITC-FBPase) and digested with subtilisin (S-FBPase). Each modification decreases the enzymes sensitivity to calcium ions.

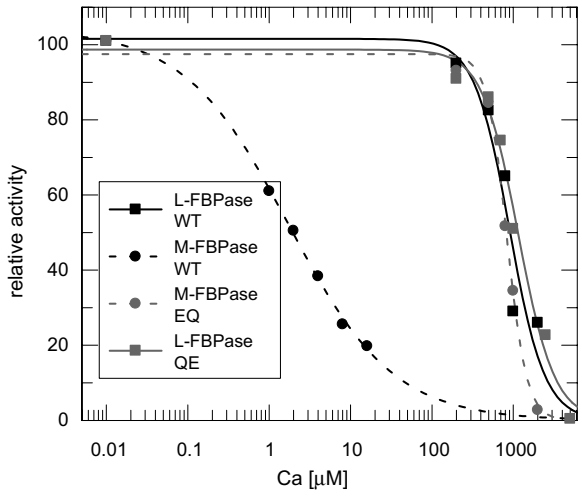

Fig. 2. The effect of calcium ions on the recombinant and mutant muscle and liver FBPases. The mutation of muscle FBPase $Glu^{69} \rightarrow Gln$ resulted in more than 500-fold increase of $I_{0.5}$ regarding the inhibition by calcium ions.

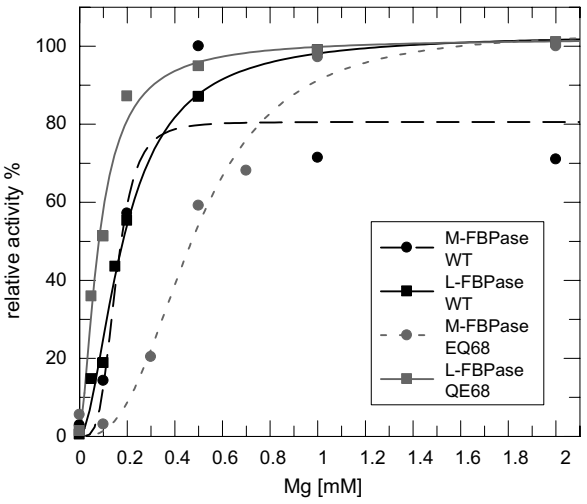

Fig. 3. The activation of the recombinant wild type and mutated FBPases by magnesium ions. Both wild type enzymes have similar sensitivity to magnesium (0.15–0.19 mM) however the mutants have either increased (liver type Gln/Glu^{69}) or decreased (muscle type Glu/Gln^{69}) sensitivity to magnesium ions.

to the loop could result in a steric hindrance precluding oscillation of the loop and therefore decreasing the FBPase sensitivity toward the inhibition with calcium ions.

The subtilisin cleaves three peptide bonds in the region of 57–67 of the liver FBPase, nevertheless the N-terminal peptide remained associated with the main protein and the enzyme was still active [21,22]. A similar effect of subtilisin has been also observed in the muscle FBPase [22]. The effect of calcium ions on the activity of the native and modified muscle FBPase is presented in Fig. 1. The sigmoidal curve of inhibition of the native FBPase by calcium ions indicates the cooperativity in calcium-enzyme binding. Muscle FBPase modified with FITC or TRITC was still active although in both cases specific activities were considerably decreased.

Comparing the region 49–78 of muscle and liver FBPases (Table 1) we noticed that in all known liver isozymes position 69 is occupied by Gln, but in muscle isozymes Gln is substituted with Glu. It prompted us to perform site directed mutagenesis of muscle Glu^{69}/Gln and liver Gln^{69}/Glu isozymes.

The effect of calcium ions on the wild type and mutants of the muscle and liver FBPase isoform are presented in Fig. 2. Like the native enzyme isolated from the muscle tissue, the recombinant muscle FBPase was highly sensitive toward the inhibition by calcium and displayed the sigmoidal curve of inhibition. Mutations also affected the affinity of muscle and the liver FBPase toward magnesium ions (Fig. 3). Mutations only slightly affected the cooperativity of the recombinant muscle and liver FBPases.

Table 2
The kinetic properties of the native rabbit muscle FBPase (N-FBPase) and the enzyme modified with fluorescein isothiocyanate (FITC-FBPase), tetramethyl-rhodamine isothiocyanate (TRITC-FBPase) and digested by subtilisin (S-FBPase) as well as the kinetic properties of the recombinant human muscle and liver FBPases: wild type (muscle: M-FBPase-WT, liver: L-FBPase-WT) and site-directed mutants in the position 69 (muscle: M-FBPase-EQ, liver: L-FBPase-QE)

	Specific activity (U/mg)	K_m F1,6P$_2$ (μM)	K_i F2,6P$_2$ (μM)	$I_{0.5}$ AMP (μM)	$I_{0.5}$ Ca^{+2} (μM)	n	Ka Mg^{+2} (μM)	n
N-FBPase	35	2.1	0.23	0.16	0.74	1.6	162	2.1
	S.E.M. 5	S.E.M. 0.2	S.E.M. 0.03	S.E.M. 0.05	S.E.M. 0.05		S.E.M. 51	
FITC-FBPase	6	2.5	0.19	0.69	247	0.2	291	1.6
	S.E.M. 3	S.E.M. 0.3	S.E.M. 0.02	S.E.M. 0.05	S.E.M. 107		S.E.M. 82	
TRITC-FBPase	2.5	2.0	0.13	2.00	>1000	–	350	1.4
	S.E.M. 0.5	S.E.M. 0.2	S.E.M. 0.02	S.E.M. 0.30			S.E.M. 74	
S-FBPase	12	2.9	0.20	0.95	>1000	–	213	1.7
	S.E.M. 1	S.E.M. 0.1	S.E.M. 0.02	S.E.M. 0.11			S.E.M. 65	
MFBPase-WT	27	2.6	0.20	0.15	2.00	1.7	154	2.2
	S.E.M. 4	S.E.M. 0.2	S.E.M. 0.03	S.E.M. 0.02	S.E.M. 0.3		S.E.M. 33	
MFBPase-EQ	26	3.1	0.35	0.21	>1000	–	484	2.0
	S.E.M. 5	S.E.M. 0.3	S.E.M. 0.04	S.E.M. 0.02			S.E.M. 45	
LFBPase-WT	28	2.7	0.25	9.00	830	2.0	188	1.8
	S.E.M. 4	S.E.M. 0.2	S.E.M. 0.02	S.E.M. 1.1	S.E.M. 150		S.E.M. 27	
LFBPase-QE	25	2.9	0.85	38.0	360	3.1	83	1.6
	S.E.M. 3	S.E.M. 0.3	S.E.M. 0.09	S.E.M. 6.2	S.E.M. 25		S.E.M. 9	

Values were estimated by least square non-linear plot of:
$v = \{V_{max} * [1 + B * (S/K_{si})]\}/[1 + (K_s/S) + (K_s/K_{si}) + (S/K_{si})]$ for K_m and K_i for F2,6P$_2$ and Hill formula for K_a of magnesium, and $I_{0.5}$ for calcium and AMP [6]; n-Hill coefficient. Hill coefficient and S.E.M. is not provided when enzymes are practically insensitive to calcium and have $I_{0.5} > 1000$ μM (S- and TRITC-FBPase, and EQ-muscle mutant). All values represent the mean and standard error (S.E.M.) of triplicate determination.

The determined kinetic properties of the native, the chemically modified and the digested with subtilisin muscle FBPases as well as the recombinant human muscle and liver FBPases have been collected in Table 2.

K_m for Fr-1,6-P_2 of muscle and liver isozyme were virtually the same and neither chemical modification nor proteolysis nor mutations have changed this parameter. K_i for Fru-2,6-P_2 of the muscle isozyme was also unchanged by chemical modification, limited proteolysis or site directed mutagenesis. The mutation of the liver FBPase only slightly affected this parameter. Chemical modification with FITC and TRITC, limited proteolysis and site directed mutagenesis (Glu^{69}/Gln) resulted in the dramatic decrease of the affinity of the muscle FBPase toward calcium ions and the slight decrease of the enzyme affinity toward magnesium ions. On the contrary, the mutation of the liver FBPase (Gln^{69}/Glu) resulted in the increase of the enzyme affinity either to calcium or magnesium ions.

The crystallographic analysis and site-directed mutagenesis [9,23,24] revealed three metal binding sites of the liver FBPase. The first site consisted of Glu^{97}, Asp^{118}, Asp^{121} and Glu^{280}. The second one consisted of: Glu^{97}, Leu^{112}, Asp^{118}, and the third one of: Asp^{68} and Glu^{97}. The same amino acid residues are present in the muscle isozyme with one exception: Leu^{112} in the liver FBPase is replaced with Lys^{112} in the muscle isozyme. It has been reported [10] that magnesium binds to the first and the second metal binding site and that potassium binds to the third site only. The ionic radius of potassium is nearly two times larger than the corresponding radius of magnesium. The ionic radius of calcium is nearly 40% larger than magnesium. Hypothetically calcium should bind to site three rather than to site one or two. In the muscle enzyme the presence of two acidic residues (Asp^{68} and Glu^{69}) interacting with calcium might stabilize the loop 52–72 in the disengaged conformation.

The crystalographic analysis and the site-directed mutagenesis are necessary to determine the muscle FBPase binding site of calcium.

Hypothetically, the single point mutation (Gln^{69}/Glu) was the crucial step in FBPase evolution enabling the regulation of glyconeogenesis in muscle cells by calcium ions.

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